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Cocultivating aerobic heterotrophs and purple bacteria for microbial protein in sequential photo- and chemotrophic reactors

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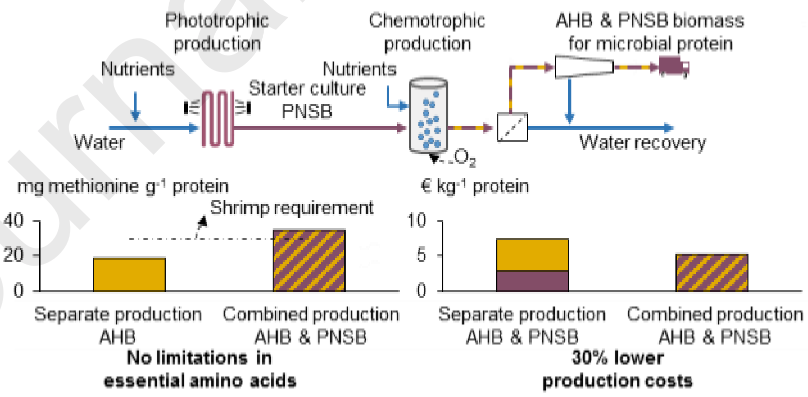
Graphical abstract



Abstract

Aerobic heterotrophic bacteria (AHB) and purple non-sulfur bacteria (PNSB) are typically explored as two separate types of microbial protein, yet their properties as respectively a bulk and added-value feed ingredient make them appealing for combined use. The feasibility of cocultivation in a sequential photo- and chemotrophic approach was investigated. First, mapping the chemotrophic growth kinetics for four *Rhodobacter, Rhodopseudomonas and Rhodospirillum* species on different carbon sources showed a preference for fructose (μ_{max} 2.4-3.9 d^{-1} 28°C; protein 36-59%$_{DW}$). Secondly, a continuous photobioreactor inoculated with *Rhodobacter capsulatus* (VFA as C-source) delivered the starter culture for an aerobic batch reactor (fructose as C-source). This two-stage system showed an improved nutritional quality compared to AHB production: higher protein content (45-71%$_{DW}$), more attractive amino/fatty acid profile and contained up to 10% PNSB. The findings strengthen protein production with cocultures and might enable the implementation of the technology for resource recovery on streams such as wastewater.

Keywords: purple phototrophic bacteria; single-cell protein; alternative protein; animal feed; aquafeeds

1 Introduction

A key challenge during the Anthropocene is to increase high-quality food production while mitigating climate change, the distortion of the biochemical nitrogen and phosphorus flows, biodiversity loss, freshwater use and land use (Pikaar et al., 2017; Steffen et al., 2015). Alternative fertilizer-to-food systems are essential (Verstraete et al., 2016), as the conventional food chain suffers from nutrient losses such as leaching, runoff and volatilization (Galloway et al., 2003). Lowering agriculture crop production through direct use of nutrients for the production of microbial biomass as a source of animal feed has the potential to increase the overall nitrogen efficiency from 4 to 10% (Pikaar et al., 2017).

This microbial biomass, so-called microbial protein (i.e. single-cell protein), can be produced with various types of yeast, fungi, algae and bacteria (Matassa et al., 2016). The production is typically performed with synthetic media from primary or renewable origin or on waste streams such as wastewater for resource recovery (Najafpour, 2015; Verstraete et al., 2016). Microbial protein production on synthetic media is mainly dominated by axenic fermenter technology, which enables culture specificity (Najafpour, 2015). On the other hand, the production of microbial protein for resource recovery is usually performed with non-axenic heterotropic cultures such as aerobic heterotrophic bacteria (AHB), purple non-sulfur bacteria (PNSB) and consortia of microalgae and AHB (Spiller et al., 2020).

AHB cultivation is the production of a consortium of bacteria under aerobic chemoheterotrophic conditions on wastewater (Vriens et al., 1989). These microbes have a high protein content (38-60 g protein 100 g^{-1} total suspended solids; TSS), an appealing essential amino acid (EAA) profile and contain several vitamins (e.g. B1, B2, B6, B12) (Vriens et al., 1989). They are mostly studied as a bulk feed ingredient, yet some studies indicate potential beneficial effects against pathogenic bacteria in aquaculture and prebiotic potential due to the presence of poly-β-hydroxybutyrate in their biomass (Crab et al., 2012). PNSB are gram-negative microbes and belong to the purple bacteria, which also comprise the purple sulfur bacteria (Blankenship et al., 1995). Purple bacteria should not be confused with the microbiological term of 'purple' for gram-positive bacteria in Gram staining. Contrary to AHB, PNSB are mainly explored in anaerobic photobioreactors (PBR) for their photoheterotrophic metabolism (Capson-Tojo et al., 2020). They have been studied for axenic cultivation on synthetic media, yet more recent literature focusses on wastewater with non-axenic cultures (Capson-Tojo et al., 2020). The main difference between PNSB and AHB is the possibility of the former for microbial selective production when cultivated under anaerobic photoheterotrophic conditions (i.e. uneven community with a high abundance of one species; (Alloul et al., 2019; Cerruti et al., 2020; Hülsen et al., 2016a; Hülsen et al., 2016b). Production of PNSB, is, however, more expensive than for AHB. Investment costs of a closed anaerobic PBR approximate € 5,000 m^{-3} compared to € 300 m^{-3} for an aerobic tank (Acien et al., 2012; van Haandel & van der Lubbe, 2012). Moreover, PNSB growth is limited by light availability for the cells, which results in lower biomass concentrations and consequently lower biomass productivities such as 4.2 g COD L^{-1} d^{-1} for photo-anaerobic membrane bioreactors (Capson-Tojo et al., 2020) compared to AHB (oxygen transfer is rate limiting, not light). The biomass of PNSB is appealing with a high protein content (40-61 g protein 100 g^{-1} TSS), an outstanding protein quality (appealing profile EAA) and vitamins such as B1, B2, B3, B5, B6, B9, E and biotin (Sasaki et al., 1998). PNSB are studied as a feed ingredient, yet they are unique due to their added-value properties beyond the nutritional content: (i) they enhance the growth performance of several fish species and shrimp (Alloul et al., 2021; Chowdhury et al., 2016; Delamare-Deboutteville et al., 2019; Noparatnaraporn et al., 1987; Shapawi et al., 2012), (ii) have antimicrobial properties against shrimp *Vibrio* pathogens as demonstrated by our previous work (Alloul et al., 2021), (iii) contain antioxidants such as carotenoids (Sasaki et al., 1998) and (iv) can have color benefits for aquaculture animals (Noparatnaraporn et al., 1987). These bacteria can also serve as an astronaut food ingredient in regenerative life-support systems (Clauwaert et al., 2017) and live or dried PNSB have added value in crop production (Spanoghe et al., 2020).

AHB and PNSB are, currently, explored as two separate types of microbial protein, yet their properties as respectively a bulk and added-value protein ingredient make them appealing for combined use. A community containing a relatively high proportion of AHB and a relatively low proportion of PNSB might be an interesting balance between high production costs of PNSB and their addition of added-value properties to the product. Obtaining a combined product is possible by producing both types of microbes in separate reactors followed by blending. However, PNSB are also able to grow aerobic chemotrophically, which, thus, in principle enables cocultivation with AHB, provided that the reactor configuration and operational conditions prevent overgrowth of one culture by the other.

This study proposes a ‘hybrid’ non-axenic photo- and chemotrophic production system. PNSB are first pre-cultivated phototrophically on synthetic medium to offer them a competitive advantage in the subsequent chemotrophic production step. Such a system requires insights in the photo- and chemotrophic PNSB growth kinetics, yet extensive knowledge of their chemotrophic growth characteristics is lacking. Several researchers have focused on axenic chemotrophic growth of pure PNSB species exploring the pigment formation during the dark and the expression of special compounds such as ubiquinone (Yen & Chiu, 2007; Zeiger & Grammel, 2010). Comparative screening of the chemotrophic growth kinetics of different PNSB species on different carbon sources is limited to *Rhodospirillum rubrum* (growth rate 3.0-3.1 d^{-1}) and *Rhodobacter capsulatus* on succinate, fructose and acetate (Schultz & Weaver, 1982; Zeiger & Grammel, 2010). An investigation of the community structure and performance of AHB seeded with phototrophic PNSB has not been explored so far.

This research aims to investigate the feasibility of the 'hybrid' system for the cocultivation of AHB and PNSB as a combined source of microbial protein. The first objective of this study was to select the most suitable PNSB inoculum, by comparing the chemotrophic growth kinetics of *Rhodobacter capsulatus*, *Rb. sphaeroides*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* on three carbon types: volatile fatty acids (VFA), alcohols, and carbohydrates. Apart from growth kinetics, the metabolic flexibility to switch from photo- to chemotrophic growth, protein content and biomass yield under chemotrophic conditions were used as performance metrics as well. The second goal was to explore (and optimize) a two-stage photo- and chemotrophic reactor system. The best PNSB from the batch tests was used as inoculum in a non-axenic semi-continuous PBR coupled to an aerobic reactor operated in batch. Effects of dissolved oxygen (DO) concentration and addition of an AHB inoculum were studied in terms of productivity, nutritional quality (protein content, essential amino and fatty acid content) and microbial community structure of the AHB & PNSB consortium.

2 Materials and methods

2.1 PNSB species

To screen for the best PNSB culture for a two-stage photo- and chemotrophic production system, six cultures were pre-selected. Four pure cultures were chosen, namely *Rb. capsulatus, Rb. sphaeroides* LMG 2827, *Rhodopseudomonas palustris* LMG 18881 and *Rhodospirillum rubrum* S1H. These species were chosen because they are one of the most studied PNSB, enabling a benchmark to previous literature (Capson-Tojo et al., 2020).

The last two selected cultures were a 3-species synthetic community (i+ii+iii) to study potential synergistic effects and an AHB inoculum originated from aerobic return sludge of a local brewery company (AB InBev, Belgium, Leuven). Axenic PNSB cultures were pre-cultivated under anaerobic phototrophic conditions with a pre-autoclaved VFA-based medium adapted from Alloul et al. (2019). The AHB inoculum was chemotrophically pre-cultivated in the same medium.

2.2 Chemotrophic growth kinetics and yield in batch incubations

Chemotrophic batch tests were divided into two experimental setups: (i) a preliminary screening was performed with nine different carbon sources in 96-Well plates and (ii) four carbon sources were selected for the second experiment in Erlenmeyer flasks based on the growth kinetics in the 96-Well plates.

The 96-Well plate experiments were performed in a working volume of 150 µL. The medium of Alloul et al. (2019) was used and the VFA were replaced by another carbon source (chemical oxygen demand basis; COD) depending on the experiment. A total of nine carbon sources were tested in triplicate containing four VFA typically used to cultivate PNSB (acetate, propionate, butyrate and a VFA mixture 1/1/1 ratio on COD basis), three carbohydrates (fructose, glucose and sucrose) and two alcohols (glycerol and ethanol) at a COD concentration of 3 g L^{-1}. In this medium, the KH_2PO_4 content was adapted to 2.7 g-P L^{-1} to cope with pH increase. The pH of the media was adjusted to 7.0 before the experiment by adding 12 M of NaOH and autoclaved (reducing sugars added after autoclaving). *Rb. capsulatus, Rb. sphaeroides, Rps. palustris* and *Rsp. rubrum* were first phototrophically pre-cultivated and then supplemented to the wells at an initial optical density of 0.200 (OD_{660nm}). Plates were then incubated in a microplate plate reader (Biotek, USA) at 28°C with vigorous orbital shaking (282 rpm) for aeration. The growth was monitored by measuring the OD_{660nm} every 2.5 h.

After the 96-Well plate pre-screening, four different carbon sources were selected, and experiments were repeated in 500 mL Erlenmeyer flasks with a working volume of 200 mL. All six cultures described in section 2.1 were tested in triplicate. The pH of the media was adjusted to 7.0 before autoclaving. The flasks were then inoculated at an initial concentration of 0.03 g TSS L^{-1}. Experiments were performed in a climate chamber (Snijders Scientific, The Netherlands) at 28°C. Flasks were covered with aluminum foil to prevent light penetration and placed on a multipoint stirrer at 300 rpm (Thermo Scientific, USA) for aeration (kLA 2 h^{-1}). The growth was monitored by measuring the absorbance at 660 nm.

2.3 Two-stage photo- and chemotrophic reactor setup and operation

The sequential system consisted of a non-axenic semi-continuous anaerobic PBR coupled to an aerobic reactor operated in batch. The main goal of the PBR was to offer PNSB a competitive advantage in the aerobic reactor.

2.3.1 Phototrophic production in a closed photobioreactor

The non-axenic PBR was a vertical tubular vessel with a working volume of 500 mL and an external diameter of 6 cm (surface to volume ratio 67 m^2 m^{-3}). It was operated at an SRT of 0.93 ± 0.1 d for 87 days at a temperature of 28 ± 2 °C, a volume exchange ratio of 54 ± 4%, illuminated with two halogen lamps at a light intensity of 30 W m^{-2} and stirred with a magnetic stirrer at 700 rpm (Carl Roth, Germany). The reactor was operated semi-continuously, by removing 250 mL effluent and adding 250 mL influent every 12h. The gas outlet was connected to a nitrogen gas expansion balloon to cope with under- and overpressure during withdrawal and fill. The reactor was inoculated with *Rb. capsulatus*, which was shown to be the most promising PNSB based on the chemotrophic batch experiments. VFA were chosen as they are the preferred carbon source for the photoheterotrophic growth of PNSB (Blankenship et al., 1995). A VFA mixture adapted from Alloul et al. (2019) was used at a 1/1/1 ratio on COD basis: 1 g acetic acid L^{-1}, 1 g propionic acid L^{-1} and 1 g butyric acid L^{-1}. The pH of the PBR was not controlled, yet the influent pH was lowered to 6.5 with 12M HCl to have a final pH of 7.0 in the effluent (pH rises due to VFA consumption). Samples were taken daily to monitor the optical density (660 nm), bacteriochlorophyll peaks (800 nm and 860 nm) to confirm the presence of PNSB, pH, temperature. The remaining sample volume was stored at -20°C for further analysis.

2.3.2 Chemotrophic production in an open aerated bioreactor

A non-axenic aerobic reactor was operated in batch until the stationary phase was reached. The working volume was 2 L and the reactor was covered with aluminum foil to prevent phototrophic growth. Stirring was done with a magnetic stirrer (Carl Roth, Germany) at 700 rpm. A pH controller (Consort, Belgium) regulated the pH between 6.9 and 7.1 through the addition of 2 M NaOH and HCl. DO concentration was controlled (Consort, Belgium) by changing the airflow. The k_La was determined through the sulfite oxidation method and was 463 ± 66 h^{-1} (Ruchti et al., 1985). The effluent of the PBR was collected as a starter culture for the aerobic reactor. The PBR effluent was first diluted 4.5 times with a fructose-based medium (most promising carbon source according to the batch tests) to a final concentration of 23 g COD L^{-1} (substrate concentration to reach 10 g TSS L^{-1} of biomass; biomass yield 0.63 g $COD_{biomass}$ g $COD_{removed}$ Figure 2). The aerobic reactor was then filled with the PBR effluent and the fructose mixture until 2 L. Per batch cultivation, 10 mL of antifoam (Antifoam silicone 414, VWR, USA) was added to the reactor to prevent foam formation (Garrett, 2017).

Five sets of experiments were performed. Biological triplicates were obtained for every experiment, based on three sequential production batches using each time fresh PBR effluent. Every batch was operated until the stationary phase was reached by monitoring the optical density at 660 nm. There was 13 ± 3% water evaporation due to aeration and heating of the reactor (28 °C). Therefore, the reactor volume was adjusted to the initial volume at the end of the experiment. Samples were then taken and stored at -20°C for further analysis.

The first experiment was inoculated with aerobic sludge to investigate the productivity and nutritional quality of AHB independently. Two subsequent experiments were inoculated with the effluent of the PBR to explore the effect of DO concentration on productivity, nutritional quality and microbial community structure of the consortium of AHB and PNSB. Two DO concentrations were tested: 0.7 ± 0.1 mg O_2 L^{-1} (experiment 'ii') and 2.0 ± 0.3 mg O_2 L^{-1} (experiment 'iii'). The COD concentration for experiments 'ii' and 'iii' was 16 g COD L^{-1}. Experiment 'iv' was inoculated with the effluent of the PBR and contained a medium with extra trace elements and a higher substrate concentration (23 g COD L^{-1}). The increased COD concentration was not an experimental variable, yet merely used to avoid substrate limitations. Experiment 'v' was inoculated with the effluent of the PBR and an additional 5% aerobic sludge to test if productivities and nutritional quality of the consortium could further be improved.

2.4 Analytic procedures

The COD was measured using photometric test kits (Merck, Germany) according to the manufacturer's instructions. The biomass yield was determined by dividing produced biomass COD by removed COD. Protein concentration was analyzed by Markwell et al. (1978) (adapted Lowry procedure). TSS and volatile suspended solids (VSS) were measured according to standard methods (Greenberg et al., 1992). Handheld meters were used to determine DO concentration (Hach, USA) and pH (Hanna Instruments, USA). Amino acids were analyzed according to the protocol described by Muys et al. (2019). All EAA profiles were normalized to the diet requirements of shrimp. This was done by dividing the individual EAA values (mg EAA g^{-1} protein) by the shrimp requirements. Values of 1 or higher indicate that the microbial protein source completely covers the shrimp requirements in terms of

EAA. Fatty acids methyl esters were prepared by direct esterification according to a modified procedure from Lepage and Roy (1984) and identified with a gas chromatograph (Toi et al., 2013).

2.5 Microbial community analyses

16S rRNA-gene amplicon sequencing analysis was performed according to De Vrieze et al. (2016) with slight modifications. In brief, DNA extraction was performed by bead beating with a PowerLyzer (Qiagen, Venlo, the Netherlands) followed by a phenol/chloroform extraction. The 16S rRNA gene V3-V4 hypervariable region was then amplified by LGC genomics GmbH (Berlin, Germany). Sequencing was performed using forward primer 341F 5'- TCCTACGGGNGGCWGCAG and reverse primer 785R 5'- TGACTACHVGGGTATCTAAKCC(Klindworth et al., 2013). Subsequently, roughly 20 ng amplicon DNA of each sample was pooled for up to 48 samples carrying different barcodes. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other mispriming products, followed by an additional purification on MinElute columns (Qiagen). Lastly, about 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries by means of adaptor ligation using the Ovation Rapid DR Multiplex System 1-96 (NuGEN) (Weithmann et al., 2016). Illumina libraries were then pooled, and size selected by preparative gel electrophoresis. Sequencing was performed on an Illumina MiSeq using v3 Chemistry (Illumina). Read assembly and cleanup were based on the MiSeq SOP described by the Schloss lab (Kozich et al., 2013; Schloss et al., 2011). In brief, mothur (v.1.40.5) was used to assemble reads into contigs, remove chimeras, perform alignment-based quality filtering (alignment to the mothur-reconstructed SILVA SEED alignment, v. 123), assign taxonomy using a naïve Bayesian classifier (Wang et al., 2007) and SILVA NR v132 and cluster contigs into OTUs at 97% sequence similarity. All Eukaryota, Archaea, Chloroplasts and Mitochondria sequences were removed. Moreover, sequences were also removed if they could not be classified at all (even at (super)Kingdom level). For each OTU representative sequences were picked as the most abundant sequence within that OTU.

2.6 Statistical analyses

Statistics were performed in R (version 3.4.1) using RStudio (RStudio®, USA) for Windows (R Core Team, 2017). The parametric analysis of variance test and post-hoc pairwise comparisons using the Tukey's range test were performed for multiple comparisons. Normality of data residuals was tested using the Shapiro-Wilk normality test and homogeneity of variances using a Levene's test. If normality was rejected, the non-parametric Kruskal-Wallis rank sum test and post-hoc pairwise comparisons using the Mann-Whitney U test (*p-values* were adjusted using the Bonferroni correction) were performed. The Welch's t-test was conducted in case of heteroscedasticity. A significance level of $p < 0.05$ was chosen.

3 Results and discussion

3.1 Chemotrophic growth kinetics and yield in batch incubations

Batch experiments in 96-Well plates (150 µL) and Erlenmeyer flasks (200 mL) were performed to determine the chemotrophic growth kinetics of PNSB. It was the objective to assess the effect of carbon source and PNSB species on the growth rate, metabolic flexibility to switch from photo- to chemotrophic conditions, biomass yield and protein content.

Growth rates of the preliminary 96-Well plate screening showed that PNSB preferred carbohydrates (growth rates $p < 0.05$) over VFA and alcohols during chemotrophic cultivation in contrast to their phototrophic metabolism where they favor VFA (Blankenship et al., 1995). More specifically, fructose resulted in significantly higher growth rates ($p < 0.05$) compared to the other carbon sources. Only *Rb. sphaeroides* showed similar growth rates for fructose, VFA and sucrose. No similar studies could be found that compared multiple PNSB species on their chemotrophic carbon preference. Imam et al. (2013) have studied 190 carbon sources to map out the metabolic and energetic network for *Rb. sphaeroides* under both photo- and chemotrophic conditions. During the experiments, only the presence or absence of growth was observed. Consequently, the authors did not derive growth kinetics.

This preliminary 96-Well plate screening allowed to select four carbon sources per species as input for the proceeding Erlenmeyer flask tests, which are presented in Figure 1. The results reconfirmed the findings of the 96-Well plate experiment, showing that fructose is an interesting carbon source for the chemotrophic cultivation of the four selected PNSB species. Tests with fructose showed the highest growth rates ($p < 0.05$; excluding *Rps. palustris*), lowest lag phase (excluding *Rsp.* rubrum), highest protein content (Figure 2; $p < 0.05$; excluding *Rb. sphaeroides*) and highest biomass yield (excluding *Rsp. rubrum).* Ghosh et al. (1994) proposed the use of a combined fructose succinate medium to enhance the pigment formation. However, fructose was not used as a tool to improve growth kinetics. In terms of yield, only Schultz and Weaver (1982) have performed a similar study for fructose, succinate and acetate using *Rsp. rubrum* and *Rb. capsulatus.* Higher biomass yields were observed for fructose (0.72-0.76 g $COD_{biomass}$ g^{-1} $COD_{catabolized}$) compared to other carbons sources such as succinate and acetate (0.50-0.62 g $COD_{biomass}$ g^{-1} $COD_{catabolized}$) for both species in line with our results.

A lag phase ranging from 4 to 49 hours was overall observed with *Rb. sphaeroides* and *Rsp. rubrum* the least metabolically flexible and *Rb. capsulatus* the most metabolically flexible to adapt from photo- to chemotrophic conditions. The 3-species synthetic community had a lower lag-phase compared to the individual species (Figure 1). Therefore, a facultative mutualistic association could have occurred within the community (Little et al., 2008). This is in contrast with photoheterotrophic PNSB growth, where competitive or antagonistic interactions were observed with negative effects on the overall growth rate (Alloul et al., 2019). The lag phase of the AHB culture was significantly lower than for the PNSB because these microorganisms did not need to switch between metabolisms. Ghosh et al. (1994) also comments that a lag phase does occur during the switch from photo- to chemotrophic conditions. It also might have been possible that the lag phase in our study was enhanced by the continuous phototrophic cultivation of PNSB. Sabaty et al. (1993) found that respiratory activity is inhibited by continuous illumination of *Rb. sphaeroides.* Similar effects are feasible for *Rb. capsulatus, Rps. palustris* and *Rsp. rubrum*. A notable observation was the formation of pigments during the dark for all PNSB species on all carbon sources. This was already discovered for *Rsp. rubrum* and is triggered by low aeration levels (Ghosh et al., 1994).

Rb. capsulatus along with fructose as carbon source was chosen for the 'hybrid' reactor experiments due to the lowest lag phase and additionally its high biomass yield and protein content.

3.2 Two-stage photo- and chemotrophic reactor cultivation

First, the results of the PBR operated semi-continuously with *Rb. capsulatus* as inoculum and a VFA based medium are described. Secondly, the results of the aerobic reactor, operated in batch mode using the PBR effluent as inoculum and a medium with fructose as a carbon source is discussed.

3.2.1 Stable phototrophic cultivation of PNSB

A non-axenic semi-continuous PBR, used as a starter culture for the aerobic reactor, was operated as chemostat at an SRT of 0.93 ± 0.1 d for 87 days. Overall, TSS concentration and protein productivity and protein content were steady overtime at respectively 1.16 ± 0.23 g TSS L^{-1}, 0.64 ± 0.11 g protein L^{-1} d^{-1} and 54 ± 2 g protein 100 g^{-1} TSS. Literature values for the protein content of *Rhodobacter* species are between 30-50 g protein 100 g^{-1} TSS, which is comparable to the PBR results in this study (Capson-Tojo et al., 2020).

Results of microbial community analysis (Figure 3) showed a high PNSB abundance (93-97%), and low diversity (Shannon index: 0.2-0.4; diversity index: 1.2-1.5). This indicates that the PBR allowed selective and stable production of PNSB overtime under phototrophic conditions, in agreement with previous literature (Hülsen et al., 2016a; Hülsen et al., 2016b). The main competitor genera were *Dysgonomonas* spp. and *Acinetobacter* spp., both gram-negative bacteria with an abundance of respectively between 0.8-3.5% and 0.4-1.7%. This is in agreement with our earlier work showing that *Acinetobacter* spp. are competitors for phototrophically cultivated PNSB (Alloul et al., 2019).

Overall, the PBR showed a stable PNSB production over time with a steady biomass concentration, protein productivity, biomass yield and PNSB community (Figure 3). The results confirm that the advantages of phototrophic cultivation are selectivity and high biomass yield (0.97 ± 0.15 g $COD_{biomass}$ g^{-1} $COD_{removed}$).

3.2.2 Chemotrophically maximizing protein productivity

Productivity and biomass yield of the aerobic reactor are presented in Figure 4. The nutritional quality was evaluated based on the EAA (Figure 5) and fatty acid profile (Figure 6). EAA were compared to fishmeal and shrimp requirements. Fatty acids were compared to fish oil. These choices were made because the authors anticipate that microbial protein will first be a substitute to aquaculture ingredients such as fishmeal (€ 2 kg^{-1} protein) due to its higher price compared to ingredients for farm animals such as soybean meal, which has a market price of 0.7 kg^{-1} protein (IndexMundi, 2019).

The results in Figure 4 compare the individual production of PNSB (PBR) and AHB (aerobic reactor) with the 'hybrid' system (i.e. aerobic reactor inoculated effluent PBR). Protein productivity was up to 10 'times higher for the 'hybrid' system (experiment ii-iv) compared to the PBR, yet biomass yield (0.53 ± 0.02 g $COD_{biomass}$ g^{-1} $COD_{removed}$) was half of that of the PBR (0.97 ± 0.03 g $COD_{biomass}$ g^{-1} $COD_{removed}$) due to aerobic oxidation of fructose to CO_2. For axenic PNSB cultures, only Zeiger and Grammel (2010) have studied chemotrophic growth of *Rsp. rubrum* and reached a productivity of 13 g TSS L^{-1} d^{-1}, slightly higher than our two-stage photo- and chemotrophic system (12 g TSS L^{-1} d^{-1}).

The individual AHB production process (experiment 'i') had a protein productivity which was 1.4 times higher (7.4 ± 0.4 g protein L^{-1} d^{-1}) compared to the experiment with the 'hybrid' system inoculated with PNSB (5.4 ± 0.6 g protein L^{-1} d^{-1}; 'iv'). AHB have a shorter lag phase than PNSB as they do not need to switch between a photo- and chemotrophic metabolism (Figure 1). However, the 'hybrid' system with the PNSB starter culture (experiment 'ii-iv') had a better nutritional quality compared to the AHB starter culture. The protein content of the experiment with the PNSB inoculum was 46-71 g protein 100 g^{-1} TSS vs. 36 ± 5 g protein 100 g^{-1} TSS for the AHB inoculum. The 'hybrid' system with the PNSB starter culture had also no limitations in EAA for shrimp (Figure 5). On the contrary, the AHB inoculum observed methionine and cysteine, and also phenylalanine and tyrosine limitations relative to shrimp requirements.

Another nutritional parameter where the 'hybrid' system (experiment 'ii-iv') outperformed the individual AHB process (experiment 'v') was the fatty acids composition (Figure 6). Experiment 'ii-iv' with the PNSB inoculum contained 6-7 g fatty acids 100 g^{-1} TSS compared to 2 g fatty acids 100 g^{-1} TSS for the AHB inoculum. Remarkably, the PBR biomass or the aerobic reactor with the PNSB starter culture were also rich in vaccenic acid (18:1(n-7)), a fatty acid already known to be abundantly present in PNSB biomass (Blankenship et al., 1995; Imhoff, 1991). However, previous literature designated 18:1 as specific for PNSB, yet our results showed that it is the fatty acid 18:1(n-7).

Experiment 'iii', at high DO concentration (2.0 ± 0.3 mg O_2 L^{-1}), showed a slightly higher protein productivity than the reactor operated at low DO levels (0.7 ± 0.1 mg O_2 L^{-1}). However, protein content was for both conditions 71 g protein 100 g^{-1} TSS ($p > 0.05$). In terms of EAA composition, values were compared to shrimp requirements. The low DO concentration showed methionine and cysteine limitations, which were not observed for the high DO concentration. This may have been linked to the higher abundance of *Rb. capsulatus* for the low DO concentration, which also showed methionine and cysteine limitations. The fatty acid profiles were comparable, and both conditions contained negligible amounts of essential fatty acids (Figure 6).

Results of the microbial community analysis showed that the 'hybrid' system enabled to produce a consortium containing a relative PNSB abundance up to 10% and around 90% for AHB (Figure 3). The highest PNSB abundance was observed for experiment 'ii', which was operated at the low DO concentration of 0.7 mg O_2 L^{-1}. Productivity results show an increase from 2.6 to 5.4 g protein L^{-1} d^{-1} for experiments 'ii' to 'iv' (Figure 4). At the same time, PNSB abundance decreased from 10% to 3%, still in line with the objectives of this research (high proportion of AHB and a low proportion of PNSB). Nonetheless, a higher abundance of PNSB is more favorable. This might be possible by acclimatizing them to oxygen, thereby, further enhancing the value of the product. PNSB have difficulties to rapidly initiate growth due to the inhibition of the respiratory activity by continuous illumination of the PBR as observed by Sabaty et al. (1993) for *Rb. sphaeroides*. Another type of PBR open to air such as a raceway reactor conventionally used for microalgae cultivation (Alloul et al., 2020), could in principle enable PNSB to adapt to oxygen and might prevent the inhibition of the respiratory activity in the subsequent chemotrophic production step. Future research should explore this.

This study shows that AHB and PNSB can be produced through a two-stage photo- and chemotrophic production system. However, cultivating AHB and PNSB separately followed by product blending might also be an option. A preliminary cost estimation based on input parameters from other work (Alloul et al., 2020; Alloul et al., 2019), showed that separately cultivating AHB (aerobic reactor) and PNSB (PBR) amounts to a production cost of respectively € 5 kg^{-1} protein and € 27 kg^{-1} protein. This would thus result in a total production cost of € 7 kg^{-1} protein considering a product of 90% AHB and 10% PNSB. On the contrary, the 'hybrid' system would result in a production cost of € 5 kg^{-1} protein or 30% lower than when the individual microbial products are blended (90% AHB and 10% PNSB). The savings for the 'hybrid' system are due to a lower PBR volume compared to an individual PNSB production process. In the two-stage process, the PBR is only used to cultivate the PNSB starter culture and the actual production occurs in the aerobic reactor. A tubular PBR contributes to 50% of total costs. Therefore, decreasing the PBR volume can significantly influence the final production costs. A thorough production cost assessment is, nonetheless, needed to further validate the benefits of the two-stage system.

4 Conclusions

Rb. capsulatus grown on fructose had the best growth performance and was, therefore, the best starter culture/carbon match for the two-stage photo- and chemotrophic systems. The biomass from the two-stage systems had an improved protein- and fatty acid content and amino acid profile (46-71 g protein 100 g^{-1} TSS; no EAA limitations; 9 g fatty acids 100 g^{-1} TSS) vs. one-stage AHB production (36 g protein 100 g^{-1} TSS; EAA limitations; 3 g fatty acids 100 g^{-1} TSS). The consortium contained up to 10% PNSB and production costs were 30% lower vs. individual AHB and PNSB cultivation followed by blending.

E-supplementary data of this work can be found in online version of the paper.

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Figure captions

Figure 1 Aerobic batch test of purple non-sulfur bacteria (PNSB) cultures and aerobic heterotrophic bacteria (AHB) showing maximum specific growth rate (left y-axis) and lag phase (right y-axis). PNSB were phototrophically pre-cultivated on a volatile fatty acid mixture. Tests were performed in Erlenmeyer flasks. Carbon sources were selected based on a 96-Well plate screening. Error bars show standard error (n=3).

Figure 2 Aerobic batch test of four purple non-sulfur bacteria showing protein content (left y-axis) as share of total suspended solids (TSS) and biomass yield expressed in chemical oxygen demand (COD; y-axis). PNSB were phototrophically pre-cultivated on a volatile fatty acid mixture. Tests were performed in Erlenmeyer flasks. Error bars show standard error (n = 3).

Figure 3 Microbial community composition, purple non-sulfur bacteria (PNSB) abundance and diversity parameters such as Shannon index and diversity index which is the exponential of the Shannon index. The photobioreactor (PBR) was inoculated with *Rhodobacter capsulatus* (in orange) and the aerobic reactor was inoculated with the effluent of the PBR and/or aerobic sludge as aerobic heterotrophic bacteria (AHB) inoculum.

Figure 4 Productivity of photobioreactor and aerobic reactor runs (left y-axis) as protein, non-protein volatile suspended solids (VSS) and fixed suspended solids (FSS) along with biomass yield (right y-axis). *Rhodobacter capsulatus* as inoculum of photobioreactor operated at 0.93 d sludge retention time and effluent of photobioreactor as starter culture of aerobic reactor. All aerobic reactor experiments were performed in biological triplicates. AHB: aerobic heterotrophic bacteria. Error bars show standard error (n= 3).

Figure 5 Essential amino acid (EAA) content in microbial biomass (g EAA 100 g^{-1} $protein_{biomass}$) relatively to juvenile shrimp requirements (g EAA 100 g^{-1} $protein_{feed}$) for the photobioreactor (Penaflorida, 1989), aerobic reactor with PBR effluent as starter culture and aerobic reactor with aerobic heterotrophic bacteria (AHB) as starter culture originating from aerobic brewery sludge. Values of 1 or higher indicate that the microbial protein source completely covers the shrimp requirements in terms of EAA. *Rhodobacter capsulatus* as inoculum of photobioreactor operated at 0.93 d sludge retention time. *Rhodobacter capsulatus* as inoculum of photobioreactor operated at 0.93 d sludge retention time.

Figure 6 (A) Fatty acid profile (left y-axis) and total fatty acid content along with of 18:1 (n-7) or 11-Octadecenoic known as a marker fatty acid for PNSB (right y-axis). A pure *Rhodobacter capsulatus* species was used to analyze fatty acids. Fish oil composition based on Trushenski et al. (2011). AHB: aerobic heterotrophic bacteria.

Highlights

(i) Aerobic grown purple non-sulfur bacteria (PNSB) prefer fructose as carbon source

(ii) *Rhodobacter capsulatus* grown on fructose had the best growth performance

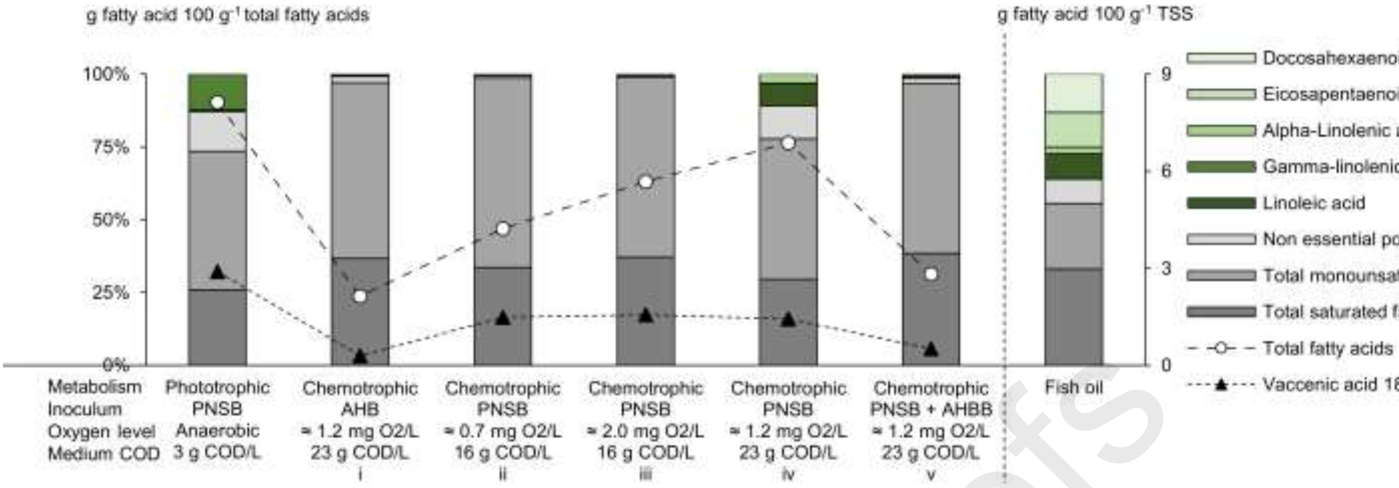
(iii) The consortium contained 10% PNSB and 90% aerobic heterotrophic bacteria (AHB)

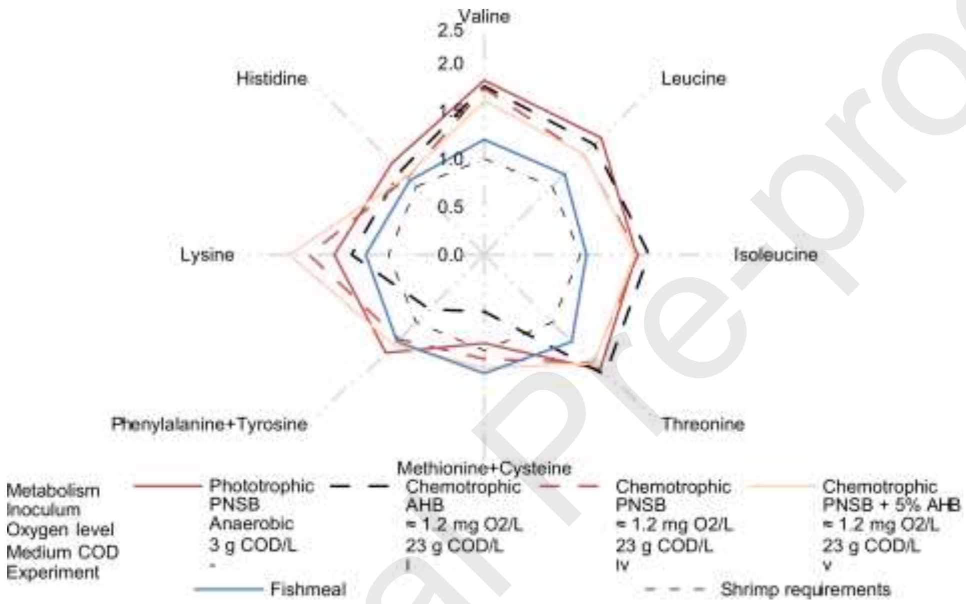
(iv) Cocultivating AHB & PNSB improved the amino acid profile vs. separate cultivation

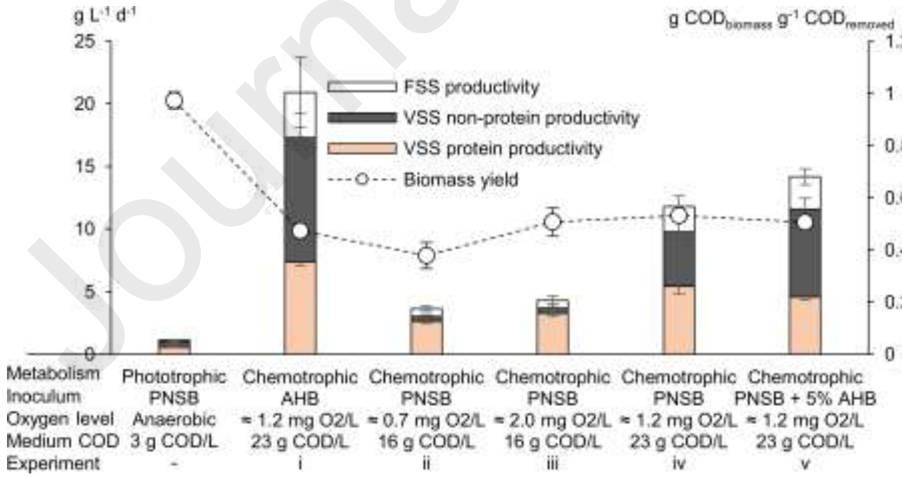
(v) Cocultivating AHB & PNSB resulted in 30% lower costs vs. separate cultivation

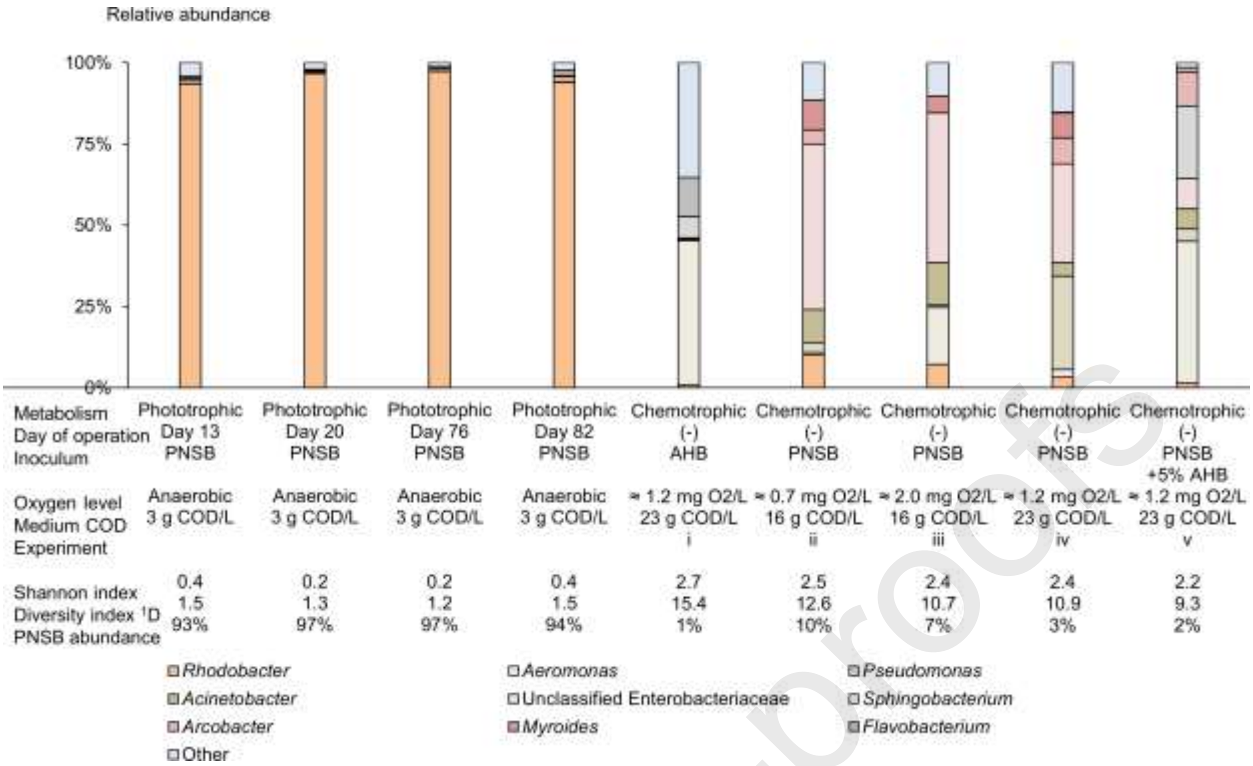
Credit Author Statement

Abbas Alloul: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration
Maarten Muys: Formal analysis; Writing - Review & Editing
Nick Hertoghs: Investigation, Formal analysis
Frederiek-Maarten Kerckhof: Formal analysis, Writing - Review & Editing
Siegfried E. Vlaeminck: Conceptualization, Supervision, Writing - Original Draft, Writing - Review & Editing


g fatty acid 100 g-1 total fatty acids
g fatty acid 100 g-1 TSS
100%
75%
50%
25%
0%
9
6
3
0
Docosahexaeno
Eicosapentaeno
Alpha-Linolenic
Gamma-linolenic
Linoleic acid
Non essential po
Total monounsat
Total saturated f
Total fatty acids
Vaccenic acid 18
Metabolism
Inoculum
Oxygen level
Medium COD
Phototrophic PNSB Anaerobic 3 g COD/L
Chemotrophic AHB ≈ 1.2 mg O2/L 23 g COD/L i
Chemotrophic PNSB ≈ 0.7 mg O2/L 16 g COD/L ii
Chemotrophic PNSB ≈ 2.0 mg O2/L 16 g COD/L iii
Chemotrophic PNSB ≈ 1.2 mg O2/L 23 g COD/L iv
Chemotrophic PNSB + AHBB ≈ 1.2 mg O2/L 23 g COD/L v
Fish oil


Valine
Leucine
Isoleucine
Threonine
Methionine+Cysteine
Phenylalanine+Tyrosine
Lysine
Histidine
2.5
2.0
1.5
1.0
0.5
0.0
Metabolism
Inoculum
Oxygen level
Medium COD
Experiment
Phototrophic PNSB Anaerobic 3 g COD/L -
Chemotrophic AHB ≈ 1.2 mg O2/L 23 g COD/L i
Chemotrophic PNSB ≈ 1.2 mg O2/L 23 g COD/L iv
Chemotrophic PNSB + 5% AHB ≈ 1.2 mg O2/L 23 g COD/L v
Fishmeal
Shrimp requirements


g L-1 d-1
g CODbiomass g-1 CODremoved
25
20
15
10
5
0
1.2
1
0.8
0.6
0.4
0.2
0
FSS productivity
VSS non-protein productivity
VSS protein productivity
Biomass yield
Metabolism
Inoculum
Oxygen level
Medium COD
Experiment
Phototrophic PNSB Anaerobic 3 g COD/L -
Chemotrophic AHB ≈ 1.2 mg O2/L 23 g COD/L i
Chemotrophic PNSB ≈ 0.7 mg O2/L 16 g COD/L ii
Chemotrophic PNSB ≈ 2.0 mg O2/L 16 g COD/L iii
Chemotrophic PNSB ≈ 1.2 mg O2/L 23 g COD/L iv
Chemotrophic PNSB + 5% AHB ≈ 1.2 mg O2/L 23 g COD/L v


Relative abundance
100%
75%
50%
25%
0%
Metabolism
Day of operation
Inoculum
Phototrophic Day 13 PNSB
Phototrophic Day 20 PNSB
Phototrophic Day 76 PNSB
Phototrophic Day 82 PNSB
Chemotrophic (-) AHB
Chemotrophic (-) PNSB
Chemotrophic (-) PNSB
Chemotrophic (-) PNSB
Chemotrophic (-) PNSB +5% AHB
Oxygen level
Medium COD
Experiment
Anaerobic 3 g COD/L
Anaerobic 3 g COD/L
Anaerobic 3 g COD/L
Anaerobic 3 g COD/L
≈ 1.2 mg O2/L 23 g COD/L i
≈ 0.7 mg O2/L 16 g COD/L ii
≈ 2.0 mg O2/L 16 g COD/L iii
≈ 1.2 mg O2/L 23 g COD/L iv
≈ 1.2 mg O2/L 23 g COD/L v
Shannon index
Diversity index ¹D
PNSB abundance
0.4 1.5 93%
0.2 1.3 97%
0.2 1.2 97%
0.4 1.5 94%
2.7 15.4 1%
2.5 12.6 10%
2.4 10.7 7%
2.4 10.9 3%
2.2 9.3 2%
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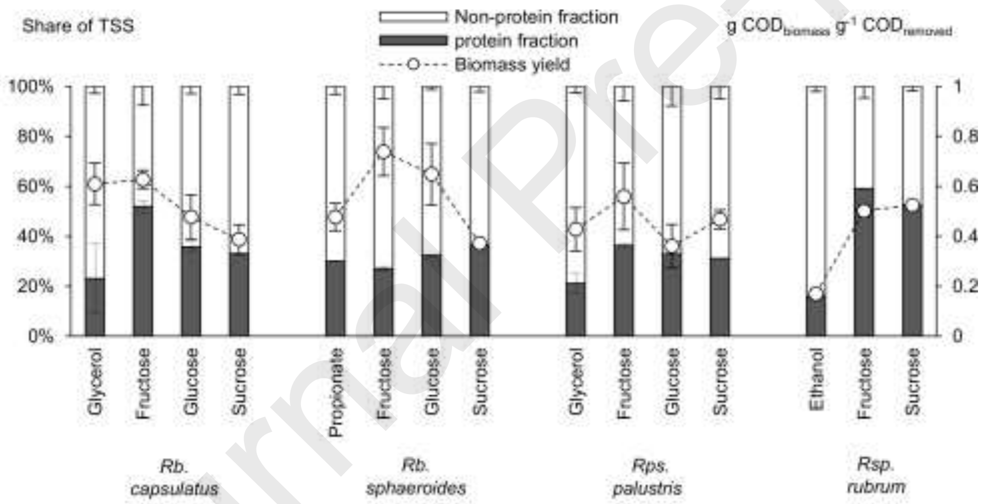
Sphingobacterium
Arcobacter
Myroides
Flavobacterium
Other


Share of TSS
Non-protein fraction
protein fraction
Biomass yield
g COD_biomass g⁻¹ COD_removed
100%
80%
60%
40%
20%
0%
1
0.8
0.6
0.4
0.2
0
Glycerol
Fructose
Glucose
Sucrose
Propionate
Fructose
Glucose
Sucrose
Glycerol
Fructose
Glucose
Sucrose
Ethanol
Fructose
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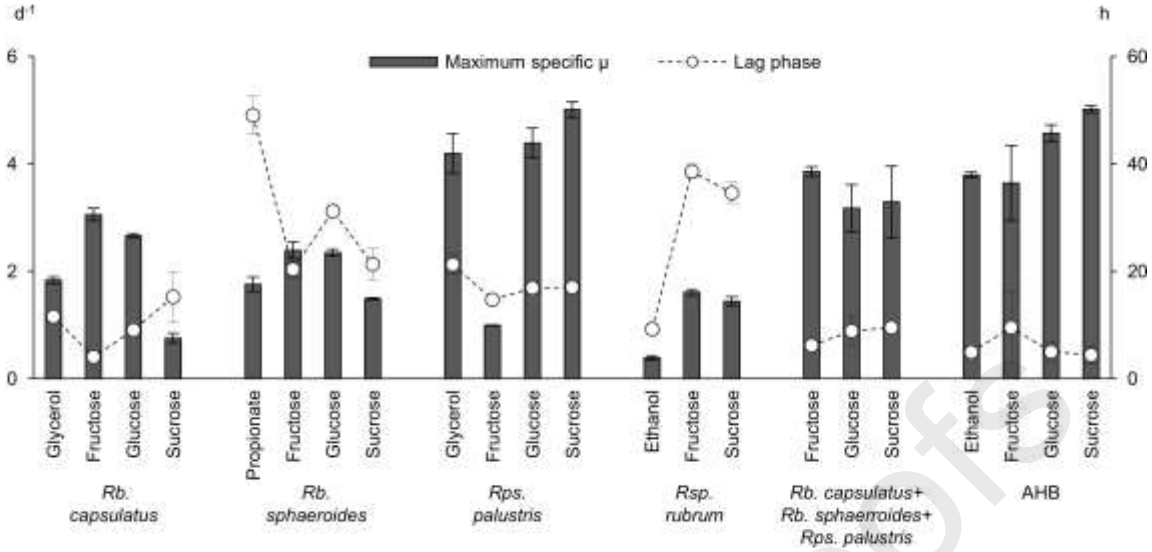
Rb. sphaeroides
Rps. palustris
Rsp. rubrum


d-1
h
6
4
2
0
60
40
20
0
Maximum specific μ
Lag phase
Glycerol
Fructose
Glucose
Sucrose
Propionate
Fructose
Glucose
Sucrose
Glycerol
Fructose
Glucose
Sucrose
Ethanol
Fructose
Sucrose
Fructose
Glucose
Sucrose
Ethanol
Fructose
Glucose
Sucrose
Rb. capsulatus
Rb. sphaeroides
Rps. palustris
Rsp. rubrum
Rb. capsulatus+ Rb. sphaerroides+ Rps. palustris
AHB